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14. ABSTRACT

The long term goals of this research are to understand the mechanisms by which NF1 controls growth using the Drosophila peripheral nerve. This system is advantageous because we can apply a number of powerful molecular genetic methodologies that are not available in other systems. The aims of this project address three specific aspects of growth control. In our first aim, we asked if NF1 acts downstream of a G protein to exert its effects. Although we have found that overexpression of amnesiac (amn) and Gas, each enhance the effects on glial growth of expression of RasV12, no equivalent effect of NF1 overexpression was observed. These data support the hypothesis that amn acts through Gas and presumably PKA to promote perineurial glial growth. However, a role for NF1 in this process is currently unclear. In our second task, we proposed to test further the hypothesis that increased neurotransmitter release from motor neurons (or increased neurotransmitter persistence) affects perineurial glial growth. All data collected for this task was negative. For task three, we found hat the formation of extra perineurial cells is conferred by either the NF1P2 null mutation or by peripheral glial RasV12 expression.

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INTRODUCTION

Over the last several years, my lab has been developing the Drosophila peripheral nerve as a system with which to identify and study the signalling pathways controlling growth of the perineurial (outer) glial layer (Yager et al., 2001). The idea behind this approach is to apply the various molecular genetic methodologies uniquely available in Drosophila to enable us ultimately to identify all of the relevant genes that interact with *NF1* to control growth, and place *NF1* and these partner genes in as complete a mechanistic context as possible. Then this mechanism could be tested and refined in systems more similar to humans but more difficult to work with (i.e. the mouse). Because all of the experimentation is performed on the acutely dissected third instar larva, there are no complications or caveats associated with experimentation on cell culture systems, and we assay the entire nerve cross section as it exists within the whole organism. We thought that a more complete mechanistic understanding of growth control within peripheral nerves would greatly facilitate the ability to design drugs able to combat neurofibromas. Within this larger context, the specific research being performed under this grant was designed to test particular hypotheses that would increase this mechanistic understanding. The first task was designed to test the hypothesis that the *amnesiac*-encoded neuropeptide acts upstream, and Neurofibromin acts downstream, of a G protein subunit. The second task proposed additional experiments to test the hypothesis that perineurial glial growth is regulated by neurotransmitter release from motor neurons. The third aim was designed to test the possibility that the regulation of perineurial glial cell size and cell number could be mechanistically uncoupled. Successful completion of these aims would provide important information concerning the control of growth within peripheral nerves at the molecular level.

BODY

Task one: Does Neurofibromin act downstream of a G protein to control perineurial glial growth? In this task I proposed to test the hypothesis that perineurial glial growth is negatively regulated by the *amnesiac (amn)*-encoded neuropeptide acting through the G protein $G_{\alpha}s$, Neurofibromin and Pushover.

<u>Does $G_{\alpha}s$ act downstream of the Amn neuropeptide to regulate perineurial glial growth?</u> The most important experiment proposed in this task was to test the prediction that the perineurial glial growth-promoting effects of the amn^{X8} null mutation would be suppressed by expression of a constitutively active $G_{\alpha}s$ called $G_{\alpha}s$*. However, we were stymied right at the outset of these experiments when we found that we could not obtain viable larvae carrying both *UAS- $G_{\alpha}s$** and amn^{X8}. When I reported this problem three years ago in my first annual report, I had no explanation for this phenomenon, but now we have the explanation: the amn^{X8} mutation, which was created by an imprecise excision of a *P(GAL4)* insertion into *amn* called amn^{28a} (Moore et al., 1998), retains the fully functional *GAL4* element that it inherited from its amn^{28a} parent. This fact was documented in my lab by both PCR (data not shown) and expression patterns and confirmed by colleagues in the field (Scott Waddell and Ulrike Heberlein, personal communication). The *GAL4* within amn^{X8} is expressed in several hundred cells within the larval central nervous system and also within the ring gland (Caldwell et al., 2005). This mutation is lethal in combination with many constitutively active, *UAS*-driven transgenes (data not shown), including *$G_{\alpha}s$**, which makes this allele essentially useless for us.

Because amn^{X8} is the only known *amn* null mutation (Moore et al., 1998), we couldn't choose another *amn* null mutation with which to replace amn^{X8}. Therefore we decided to remove the *GAL4* element from amn^{X8} by passing amn^{X8} once again through P-element mediated transposition, and selecting for amn^{X8}-derivative chromosomes that had lost their lethality in combination with one of the *UAS*-driven, constitutively active transgenes (*UAS-Ras^{V12}*, in this case). I believe that we have obtained such an excision, and if verified by PCR, it will be back-crossed to our isogenic wildtype stock for isogenization and then introduced into *gli-GAL4* for crossing to *UAS-$G_{\alpha}s$** for analysis as described in the grant proposal.

Effects of overexpression of the genes of the protein kinase A pathway on perineurial glial growth: The other experimental approach proposed in this task was to test the possibility that overexpression of either *amn*, a constitutively active $G_{\alpha}s$ called $G_{\alpha}s$*or *NF1* should each hyperactivate PKA and thus enhance the effects of Ras^{V12} on growth. Furthermore, this hypothesis suggests that the $NF1^{P2}$ null mutation should be epistatic to overexpressed *amn* and $G_{\alpha}s$*, but that overexpressed *PKA** should epistatic to $NF1^{P2}$. Last year I presented data showing that overexpression of *amn*, $G_{\alpha}s$*, and *NF1* did indeed enhance the effects of Ras^{V12}. More recently we found that the effects of $NF1^{P2}$ were indeed epistatic to overexpression of *amn* (the enhancement of Ras^{V12} by overexpression of *amn* was lost in an $NF1^{P2}$ mutant background: perineurial glial thickness in larvae overexpressing both Ras^{V12} and *amn*, but in an $NF1^{P2}$ mutant background, was 1.79 +/- .08, n=27, which is identical to what is observed in the absence of overexpression of *amn*, but significantly reduced compared to what is observed in an $NF1^{+}$ background). Furthermore, we found that, as predicted, overexpression of *PKA** was indeed epistatic to $NF1^{P2}$: perineurial glial thickness in larvae overexpressing both Ras^{V12} and *PKA**, but in an $NF1^{P2}$ mutant background, was 5.36 +/- .54, n=16, which is much thicker than perineurial glial thickness in the absence of *PKA**.

During the previous year, we have taken two approaches to test further these preliminary observations. First, we repeated most of the experiments described above to test reproducibility, increase the number of replicates, and increase the statistical significance of any positive result. Second, to rule out the possibility that any effect observed resulted from genetic background effects, rather than specific effects of transgenes, we are backcrossing at least five times all stocks carrying transgenes or mutations of interest to our isogenic wildtype stock, thus placing all experimental and control larvae in the same genetic background. Although most of the backcrossing is complete, measurements of perineurial glial thickness in these isogenized larvae is still in progress. Stock isogenization is essential for proper data collection in this system, because this system (particularly in a *push* mutant or Ras^{V12}-expressing background) appears to be exquisitely sensitive to genetic background effects. Andre Bernards, studying the role of *NF1* in body size regulation, is also finding strong effects of genetic backgrounds. These effects might be the Drosophila equivalent of the well-known susceptibility to genetic modifiers of human patients with Neurofibromatosis.

When measurements on the same genotypes were repeated, we found that we could not reproduce some of the effects observed previously. In particular, we no longer observed a statistically significant effect of *NF1* overexpression in combination with the strong Ras^{V12} (compare lanes #3 and #5 in Figure 2 below). This result suggests that *NF1* overexpression does not hyperactivate the PKA pathway. This conclusion should by no means be taken to suggest that *NF1* is not an intermediate in the PKA pathway. Genetic overexpression is not expected to confer any phenotype even for genes involved in particular processes if the amount of the gene product is not limiting. A more definitive result will come from the ability or inability of $NF1^{P2}$ to suppress the effects of Ras^{V12} in our isogenized lines. These larvae are currently under analysis.

In addition, the effects of overexpression of $G_{\alpha}s$* in combination with the weak Ras^{V12} (compare lanes #4 with lane #6 in Figure 1 below) also lost statistical significance. However, significant effects were still observed with overexpression of *amn* in combination with the weak Ras^{V12} (p=0.0094, compare lanes #4 and #5, Figure 1 below), and with overexpression of $G_{\alpha}s$* or *amn* with the strong Ras^{V12} (Figure 2 below, compare lane #3 with lanes #4 and #6). Most encouragingly for the hypothesis put forward above, overexpression of *amn* significantly enhanced the effects of the strong Ras^{V12} even in our isogenized lines (data not shown).

These observations suggest that the Amn-PKA hypothesis described above might still be true but I would like to perform the following experiments prior to publication to extend and confirm these observations. First, I note that from this observation, the somewhat surprising conclusion is that the source of Amn neuropeptide in the peripheral nerve is not the motor neuron, as anticipated, but rather the peripheral glia itself. This conclusion comes from the observation that the effects of *amn* overexpression are observed when *amn* is overexpressed in the peripheral glia. The amn neuropeptide released from the peripheral glia apparently acts cell autonomously on Amn receptors in the peripheral glia. Presumably, Amn is released from the peripheral glia as a result of increased peripheral glial [Ca^{2+}]; this increased [Ca^{2+}] might result from neurotransmitter released from the motor neuron acting on the peripheral glia. This potentially important observation needs to be confirmed with immunocytochemical methods; such experiments are currently underway but are beyond the scope of this grant. Second, we need to confirm these observations for transgenes overexpressing *G□s** or *PKA* in isogenized lines. Third, we need to repeat the epistasis tests described above, particularly those involving *$NF1^{P2}$*, in isogenized lines, which have mostly been constructed and are currently under analysis. If these results are similar to what we have observed in our non-isogenized lines, then our hypothesis will be proven. Fourth, I would like to identify the PKA-regulated step in the growth control pathway (e.g. does PKA activate growth via phosphorylation of Akt in the C terminus). This last step is beyond the scope of the current grant.

Figure 1: Enhancement of the effects of a weak *Ras^{V12}* by overexpression of *amn* and *G□s**

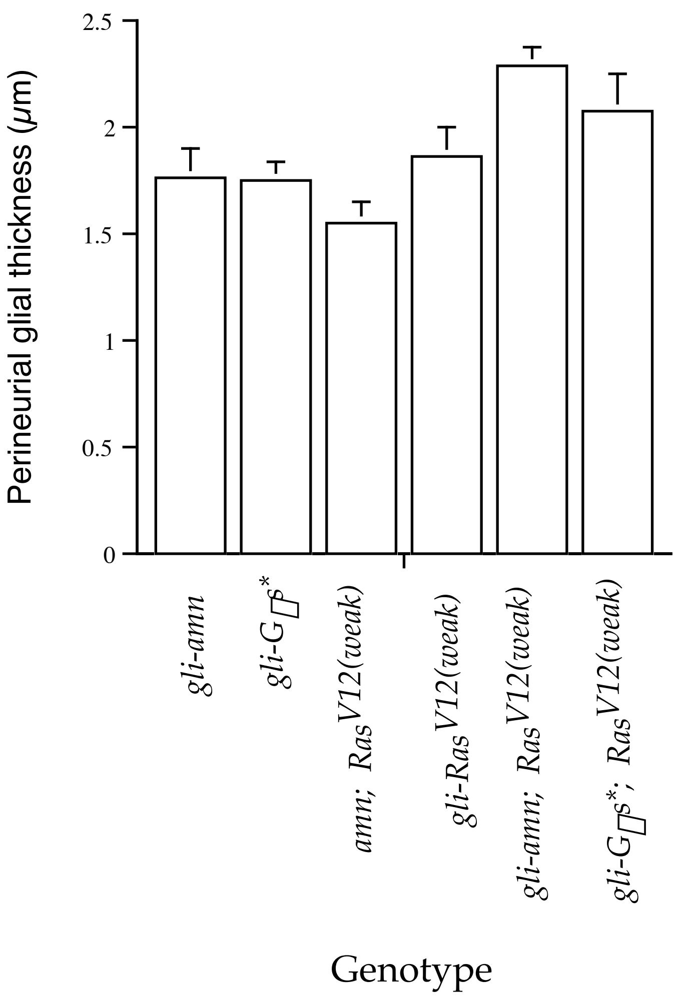

Figure 1: Perineurial glial thickness (Y axis) in larvae from the indicated genotypes (X axis). Means +/- SEMs are indicated. The following pairwise combination had a statistically significant difference (two-tailed, unpaired t test): for *gli-$Ras^{V12(weak)}$* (n=49), lane #4, vs. *gli-amn; $Ras^{V12(weak)}$* (n=47), p=0.0095.

Task two: Further tests of the hypothesis that increased neurotransmitter release from motor neurons (or increased neurotransmitter persistence) affects perineurial glial growth. We constructed and analyzed two of the fly lines that we proposed. These lines are: *eag Sh; NF1* and *eag; ine; NF1*. In data reported in my first annual report, we found that perineurial glial growth was not significantly affected in each triple mutants compared to the double mutants assayed previously. Thus, we were not able to demonstrate that increased neurotransmitter signalling from the motor neurons activates perineurial glial growth. This year, we constructed the *eag Sh; push* triple mutant for analysis, as proposed in the statement of work. Unfortunately, the triple mutant is so sick that no larvae homozygous or hemizygous for each of the three mutations could be obtained, so no data from this genotype could be collected.

Figure 2: Enhancement of the effects of a strong *Ras*^{V12} by overexpression of *amn, NF1* and *Gαs*

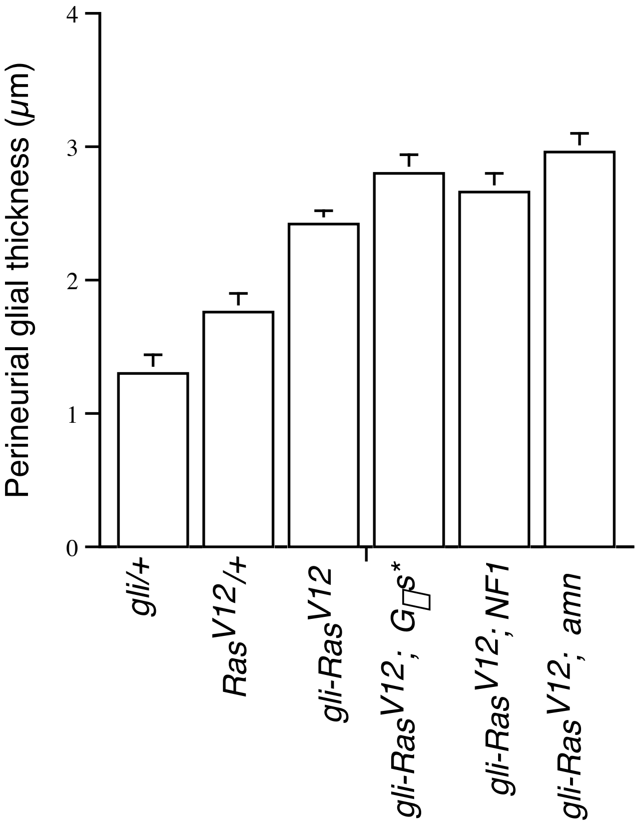

Figure 2: : Perineurial glial thickness (Y axis) from the indicated genotypes (X axis). Means +/- SEMs are indicated. The following pairwise combinations had statistically significant differences (two-tailed, unpaired t test): *gli-Ras*$^{V12(strong)}$ (n=72), lane #3, vs. *gli-amn; Ras*$^{V12(strong)}$ (n=48), lane #6, p=0.0021; for *gli-Ras*$^{V12(strong)}$ (n=72), lane #3, vs. *gli-Gαs*; Ras*$^{V12(strong)}$ (n=48), lane #4, p=0.03.

So far, all of the data that we have managed to collect for this task has been negative: we have been unable to observed the specific increases in perineurial glial growth that we anticipated. Why? The hypothesis was based on the assumption that neuronal activity would affect the activity of Ras and its downstream effectors. Although this hypothesis has by no means been disproven, and tests of this hypothesis are still underway (described below), it is worthwhile at this point to begin to think of alternative hypotheses. Given our discovery that the growth-promoting effects of *PI3 kinase* overexpression are much stronger than the effects of *Ras*^{V12} overexpression (data obtained from my other DOD grant, to be reported in Lavery et al., in preparation), I would like to raise the possibility that neuronal activity is promoting PI3 kinase activity in a Ras-independent manner.

To begin to address this possibility, we successfully constructed flies carrying *eag Sh* and *gli-GAL4* in an isogenized background. Now we will be able to cross flies from this line with flies carrying any *UAS*-driven transgenes, such as *UAS-PKA** or *UAS-Ras*^{V12}, and measure glial thickness in larval progeny. This analysis will enable us to evaluate the effects on perineurial glial growth of activating specific peripheral glial-signalling pathways in a hyper-excitable larval background. In this way, we should be able to test the hypothesis that neuronal activity is necessary, but not sufficient, to activate the PI3 kinase pathway in a Ras-independent manner.

Task three: Can perineurial glial growth be genetically uncoupled from perineurial glial proliferation? We counted the number of perineurial glial nuclei per length of nerve from several mutants exhibiting increased glial growth as well as their wildtype controls, as described in the statement of work. In my first annual report, I showed that expression of *Ras*^{V12} in the peripheral glia increased perineurial glial nuclear density by about 50%. This observation has been confirmed in larvae from isogenized fly lines: in fact, our final, publishable value is that *Ras*^{V12} expression in peripheral glia doubles perineurial glial nuclear density (see Figure 3 below). This finding will be reported in Lavery et al. (in preparation). Of even greater interest, we also found that in properly isogenized fly lines, the *NF1*^{P2} null mutation also doubles the number of perineurial glial nuclei (Figure 3). It is possible that *NF1*^{P2} increases perineurial glial nuclei number by activating Ras in the peripheral glia. We are currently constructing the fly stocks necessary to test if *NF1* is required in the peripheral glia, or in other tissues, for proper control of nuclei number. We have successfully constructed isogenic *ine push* double mutant lines and are currently measuring nuclear density in this double mutant.

Figure 3: *NF1*^{P2} and peripheral-glial expression of strong *Ras*^{V12} increases perineurial glial nuclei number

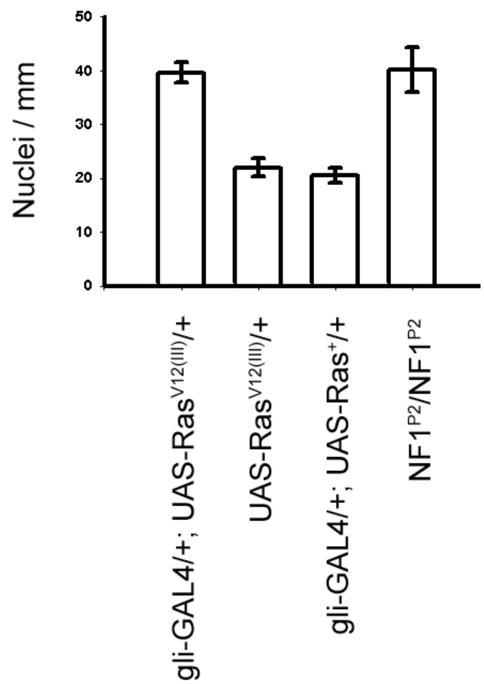

Figure 3: *NF1*^{P2} and peripheral-glial expression of strong *Ras*^{V12} increases perineurial glial nuclei number. Means +/- SEMs are indicated. Nuclei from at least 19 nerves were counted. The following genotypes exhibited significant differences (Student's unpaired t-test) in nuclei/mm: *gli-GAL4/+; UAS-Ras*^{V12}/+ (n=64 nerves) vs. *UAS-Ras*^{V12}/+ (n=19 nerves), p<0.0001, and vs. *gli-GAL4/+; UAS-Ras*$^{+}$/+ (n=20 nerves), p<0.0001; *NF1*^{P2}/*NF1*^{P2} (n=19 nerves) vs. *UAS-Ras*^{V12}/+, p=0.0003, and vs. *gli-GAL4/+; UAS-Ras*$^{+}$/+, p<0.0001. *NF1*^{P2}/*NF1*^{P2} vs. *gli-GAL4/+; UAS-Ras*^{V12}/+ was judged not significantly different (p=0.8978)

INDIVIDUALS WHO HAVE BEEN FUNDED BY THIS GRANT

Elizabeth Carter
April Ewing
Veronica Hall
Angela Lynn
Magdalena Walkiewicz
Michelle Wells

KEY RESEARCH ACCOMPLISHMENTS

We have evidence that the *amnesiac*-encoded neuropeptide, and $G_{\alpha}s$* enhance the effects of Ras^{V12} on perineurial glial growth. This discovery supports the hypothesis of task one that Amn acts via the $G_{\alpha}s$ protein in the control of perineurial glial growth (Task One).

We have evidence that Amn, $G_{\alpha}s$, and Neurofibromin activate PKA in Drosophila peripheral glia (Task One).

Our results for Task two were negative and there were no key research accomplishments.

We showed that expression of Ras^{V12} in the peripheral glia increases perineurial glial nuclear density (nuclei per mm of nerve). This result demonstrates that $\mathrm{Ras}^{\mathrm{V12}}$ nonautonomously increases perineurial glial cell proliferation or recruitment of mesodermal precursors into the perineurium, as hypothesized (Task 3) We have found a similar phenotype in the $NF1^{P2}$ null mutant.

REPORTABLE OUTCOMES

1. Presentation entitled " Ras activity in peripheral glia promotes perineurial glial growth in Drosophila peripheral nerves", by James C. Yager, Alexander Rottgers, Michelle C. Wells, Elizabeth L. Carter and Michael Stern, was presented in a platform session at the NNFF International Consortium meeting, held at Aspen, CO, in June, 2003.

2. Presentation entitled "Evidence that PI3 kinase mediates the effects of Ras on perineurial glial growth in Drosophila peripheral nerves" by William Lavery, Michelle C. Wells and Michael Stern was presented in a platform at the NNFF International Consortium meeting, held at Aspen, CO, May 23-May 25, 2004. Although not in evidence from the abstract title, during the first half of the talk I presented the data on interactions between Ras^{V12} and overexpression of *amn* and $G_{\alpha}s$* as described in the "Body" section above, under "Task one".

CONCLUSIONS

I report three major conclusions. First, I report that overexpression of *amn* enhances perineurial glial growth in larvae expressing Ras^{V12}. This effect is observed even with isogenized lines, demonstrating that this effect is due to the *amn* overexpression and not to a genetic background effect. A similar enhancement is observed with overexpression of $G_{\alpha}s$* and *PKA*. However these effects have not yet been tested with isogenized lines, so in these cases, the conclusion should be considered tentative. Taken together, these observations support the hypothesis that activation of the Amn- $G_{\alpha}s$ -PKA pathway enhances the effects of Ras^{V12} on perineurial glial growth. These molecules could ultimately serve as targets for therapeutic manipulation of peripheral nerve growth. Currently we don't know how this pathway impinges on the Ras/Raf/PI3K pathway to regulate growth; our next task, which is beyond the scope of this current grant, will be to address these issues.

Second, we also provided evidence that the $NF1^{P2}$ null mutation suppresses the effects of Ras^{V12}, was epistatic to overexpression of *amn* in this suppression, but overexpression of *PKA* was epistatic to $NF1^{P2}$ for this effect (see Body of report, Task One, above). These effects have not yet been confirmed with isogenized lines, so these conclusions should be considered tentative. However, if these conclusions are finally proven, then these results would demonstrate that Neurofibromin has two, opposing roles in the regulation of perineurial glial growth. An understanding of the signals regulating the activity of Neurofibromin will not only add to our general knowledge of nerve growth control, but also improve our ability to select useful pharmacological agents for treatment.

Our third accomplishment is our demonstration under Task Three that in properly isogenized fly lines, the $NF1^{P2}$ mutation increases perineurial glial growth. Furthermore, larvae with peripheral glial expression of constitutively active Ras^{V12} also increase the number of perineurial glial cells. In my opinion, this is the best-supported and most important discovery of the grant: given that Ras^{V12} expression in the peripheral glia only modestly increases perineurial glial thickness, and because the $NF1^{P2}$ mutation has no significant effect on perineurial glial thickness (Yager et al., 2001; note that this measurement was performed in a different genetic background), these data confirm that mechanisms that regulate perineurial glial growth can be genetically uncoupled, at least in part, from mechanisms that regulate perineurial cell number.

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APPENDIX

1) Abstract of presentation to the NNFF Consortium on NF1 and NF2 (Aspen, CO, June, 2003).

2) Abstract of presentation to the NNFF Consortium on NF1 and NF2 (Aspen, CO, May, 2004).

ABSTRACT

TITLE: Ras activity in peripheral glia promotes perineurial glial growth in Drosophila peripheral nerves

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Drosophila peripheral nerves comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). It was previously shown that perineurial glial growth in third instar Drosophila larvae is negatively regulated by a number of genes including *push*, which encodes a large Zn^{2+}-finger-containing protein, *amn*, which encodes a putative neuropeptide, *ine*, which encodes a putative neurotransmitter transporter, and *NF1*. We show that mutations that reduce Ras activity suppress the increased perineurial glial thickness of the amn^{X8} deletion mutant and the *ine;* $NF1^{P2}$ and *ine push* double mutants. In contrast, expression of the constitutively active Ras^{V12} mutation specifically in the peripheral glia is sufficient to confer increased perineurial glial growth. We also show that the effect on perineurial glial growth of Ras^{V12} is significantly enhanced by mutations in *push* but not by mutations in *ine* or *NF1*. The *push* mutant, but not the *ine* or *NF1* mutants, also exhibits hypersensitivity to low levels of Ras^{V12} expression. We conclude that Ras activity is both necessary and sufficient for increased perineurial glial growth, and that Ras can promote perineurial glial growth cell-nonautonomously. We further suggest that mutations in *NF1* and *ine*, but not *push*, increase perineurial glial growth by increasing [Ras-GTP]. Mutations in *push* could act on a pathway parallel to Ras, or increase Ras signalling independently of an effect on [Ras-GTP]. Cell nonautonomous effects of Ras activity could be responsible for the cellular heterogeneity of neurofibromas.

ABSTRACT FORM

TOPIC: Signaling pathways in NF and TSC

TITLE: Evidence that PI3 Kinase mediates the effects of Ras on perineurial glial growth in Drosophila peripheral nerves

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Drosophila peripheral nerves comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). We have been using these nerves as an assay platform to test the effects of mutations and transgenes on perineurial glial growth. It was previously shown that perineurial glial growth in third instar larval nerves is regulated by a number of genes including *push*, which encodes a large Zn^{2+}-finger-containing protein, *amn*, which encodes a putative neuropeptide related to PACAP, and *NF1*. We found that expression of the constitutively active Ras^{V12} transgene specifically in peripheral glia increased growth within the perineurial glia. This result demonstrates that Ras activity is sufficient to promote perineurial glial growth, and that Ras can act cell nonautonomously. Surprisingly, we found that the $NF1^{P2}$ null mutation suppresses these effects of Ras^{V12}, suggesting that *NF1* has a relevant activity that promotes, rather than inhibits, perineurial glial growth. The possibility that activation of adenylate cyclase represents this second activity is supported by the observation that expression within peripheral glia of any of three genes expected to increase protein kinase A (PKA) activity (a constitutively active PKA, the *amn*-encoded PACAP-like neuropeptide, or a constitutively active $G_{\square}s$) strongly enhances the growth promoting effects elicited by Ras^{V12} alone. These results are consistent with the possibility that a signalling pathway from the Amn neuropeptide through $G_{\square}s$, Neurofibromin, and PKA strongly potentiates the effectiveness of constitutive Ras activity on perineurial glial growth.

To identify the downstream components that mediate the effects of Ras, we tested the effects of constitutively active *Raf* and *PI3 Kinase* transgenes on perineurial glial growth. We found that expression of a constitutively active *PI3 Kinase,* but not a constitutively active *Raf*, strongly increased perineurial glial growth, suggesting the possibility that PI3 Kinase is an important mediator of the growth-promoting effects of Ras in peripheral nerves.